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Patents ADP number *(if you know it)* 59600 7001

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Selection System

The invention relates to a selection system which permits the selection of polypeptides displayed in a phage display system.

Viruses have been used for the display of peptides and proteins [26, 21, 44]. In particular filamentous bacteriophage have been used for display of proteins and peptides by fusion of the genes encoding the proteins or peptides to the gene encoding a phage coat protein. As the fusion gene is encapsidated in the phage that is displaying the fusion protein, this provides a linkage of phenotype and genotype. Repertoires of proteins can be encoded by a population of phage, and the rare phage encoding proteins with predefined binding activities isolated by binding to solid phase. In this way synthetic human antibodies of predefined antigen-binding specificity have been selected from repertoires of antibody fragments assembled from different structural elements [10]. As the antibody needs to be folded to bind antigen, selection for binding also selects for folding. This principle has also been used for selection of folded peptides where binding is mediated by a discontinuous epitope [8, 11-13].

A problem present in phage display systems is the presence of high levels of background caused by the presence of phage not encoding desired polypeptides. For example, antibody repertoires are commonly encoded as fusion proteins with the p3 protein on phagemid vectors and are encapsidated by the use of helper phage. The helper phage coat protein competes with the fusion of antibody and coat protein (encoded on the phagemid), leading to phage with "monovalent" rather than multivalent display of folded antibody fragments. This can be useful in discriminating between the affinity and the avidity (with multivalent display) of the antibodies displayed on the phage. However the great majority of phages only display the helper phage coat protein which contributes to a "background" binding to antigen. In this case it is desirable to select for phages that display folded antibodies, and to eliminate those that do not.

Moreover, all of the systems in current use rely on a binding activity in the polypeptide to be selected in order to perform the isolation of the desired display bodies from those which do not encode polypeptides having a desired binding activity. This places a limitation on available display systems to the selection of folded polypeptides which possess a binding activity. It would be desirable to have a means for selection of displayed proteins or polypeptides that is independent of the binding activity thereof.

For example, there is considerable interest in building folded proteins *de novo*. Attempts have been made to design proteins *de novo* by assembly of predefined elements of secondary structure and also from random sequences (for review [5]). In some cases the designed proteins have been shown to retain elements of secondary structure but lack the stable tertiary interactions characteristic of the folding of native proteins, suggesting the presence of molten globules (see [6] and references therein). More successful has been the creation of native-like proteins based on a pre-existing backbone [7, 8]. In these cases the binding activities of a *de novo* designed protein will be unknown. In this case it is desirable to select for phages displaying folded proteins, and to eliminate those that do not.

Attempts have been made to screen for folded proteins by their ability to survive degrading enzymes in bacteria [16-18]. However, because proteins in bacteria are not subject to the powerful selection forces which can be used with phage display, such techniques are not suitable for large-scale selection of a repertoire of polypeptides.

Summary of the Invention

The present invention is based on the novel principle that the introduction of polypeptides comprising protease cleavage sites into the sequence of the viral coat protein, and exposing the virus to a protease such that the protease cleavage sites are cleaved and the viral coat protein is unable to mediate infection can provide a powerful means for selection for displayed folded polypeptides that is independent of binding of the phage to solid phase.

According to a first aspect, therefore, the invention provides a method for the selection of a virus comprising the steps of:

(a) introducing a polypeptide comprising a cleavable site into the sequence of a viral coat protein; and

(b) exposing the virus to a cleaving agent such that the cleavable site is cleaved and the viral coat protein is unable to mediate infection of the virus.

Preferably, the method further comprises the step of :

(c) selecting those viruses which remain infective.

The method according to the invention may be used to decrease background in a phage selection experiment, particularly in the case where defective virus-based episomes, lacking all the viral proteins necessary for making intact virus, are used in connection with helper virus. Alternatively, in a preferred embodiment, the method according to the invention may be employed for the specific selection of proteins or polypeptides according to their ability to fold correctly and thus mask, or otherwise make unavailable, a cleavable site.

The invention moreover provides virus comprising a cleavable site in a coat protein, and polypeptide libraries encoded as inserts in the coat proteins of viruses and incorporating a cleavable site, in accordance with the invention.

Brief Description of the Figures

Figure 1. Cleavage of phage containing protease sites. Phages are prepared by rescue with KM13 (pHEN1, A + B), or with VCSM13 (pK1, C + D). Uncleaved (A + C) or cleaved with trypsin (B + D). 5 μl, 2.5 μl and 1 μl phages are loaded as indicated. Molecular weight markers are in Kd.

Figure 2. The phagemid vectors pK1 and pK2. These vectors contain a protease cleavable sequence between D2 and D3 of the phage p3 protein. In pK1, D2 + D3 are in frame; in pK2, D3 is out of frame.

Figure 3. Binding of phage-barnase to barstar. Phage displaying different fusion protein are incubated with biotinylated barstar captured on streptavidin-coated plate and detected by ELISA. a) barnase mutant A, b) barnase mutant B, c) villin , d) no phage.

Figure 4. Temperature denaturation of phage fusion proteins. Phagemids are rescued with KM13, infectivity (TU/ml) shown after incubation and cleavage with trypsin at given temperatures. Fusion with (▲) villin subdomain, (◆) barnase mutant A, (□) barnase mutant B, (○) chloramphenicol resistant pHEN1.

Detailed Description of the Invention

Definitions

Virus According to the present invention, virus may be selected by cleavage of non-resistant virions using a cleaving agent. As used herein, "virus" refers to an infective inocculum of virions, which may incorporate cleavage sites, optionally as part of heterologous polypeptides encoded by the viral genome. Thus, "virus" may refer to a plurality of virions, such that it may encode a repertoire of polypeptides; alternatively, as the context requires, it may be used to denote a single virion. The term "virus" includes any suitable virus which may incorporate a cleavage site, either naturally or through manipulation. A preferred virus for use in the present invention is bacteriophage, preferably filamentous bacteriophage.

Polypeptide The term "polypeptide" is used generally to denote polypeptides which are incorporated into viral coat proteins. In one sense, polypeptide is used interchangeably with "protein" herein and denotes a displayed polypeptide which is displayed by the virus for selection purposes. Displayed polypeptides encoded in the

virus of the invention as fusions with virion polypeptides are preferably heterologous polypeptides which are potentially capable of folding to a folded conformation whilst incorporated in the virion polypeptides. Substantially any polypeptide capable of folding may be selected for by the method of the present invention, including structural polypeptides, polypeptides having enzymatic activity and polypeptides having binding activity, including antibodies and antibody fragments. Cleavage sites may be present in the polypeptides, and may be naturally-occurring or may be engineered into the polypeptide or into a linker peptide attached thereto. "Polypeptide" may also refer to inserted polypeptides which are essentially non-folding polypeptides and serve to encode a cleavable site and insert this site into the coat protein of a virus. Inserted polypeptides may take the form of N- or C-terminal fusions, or may form part of the coat protein itself.

Cleavable site A site capable of cleavage when exposed to a cleaving agent. In the present invention, the use of protease cleavage sites, capable of being cleaved with proteases, is preferred. Protease cleavage sites may be part of, or incorporated in, polypeptides according to the invention; alternatively, it may be independently engineered into the coat protein of the virus. A feature of the cleavable site is that it should either be absent from the virus other than at the site of its specific insertion according to the present invention, or otherwise inaccessible to cleavage, or present only in viral proteins which are not required after virion assembly to mediate infection.

Repertoire A repertoire is a collection of members, preferably polypeptides, which differ slightly from each other in a random or partially randomised manner. Preferably, a repertoire of polypeptides is a collection of variant polypeptides which preferably incorporate random or partially randomised mutations. As used herein, a repertoire preferably consist of 10^4 members of more. A repertoire advantageously comprises a very large number of members, typically between 10^8 and 10^{11}, and potentially 10^{14} or higher. From a repertoire, the invention allows the selection of members which have the ability to fold, preferably into an active conformation.

Phagemid A phagemid is a plasmid cloning vector which comprises viral replication sequences but is deficient in at least one viral function. This means that whilst phagemid may be inserted into host cells by conventional nucleic acid transfer methods, and will exist in the host cells in an episomal state, they are unable to assemble into virions and thus complete a viral cycle of infection. Helper phage are used to supply the deficient viral functions and permit the phagemid to be packaged into virions. In accordance with the invention, phagemid may encode coat protein fusions with heterologous polypeptides which incorporate a cleavable site.

Helper phage Helper phage provide the viral function lacking in phagemid in order to allow packaging of the phagemid into virions. According to the invention, helper phage may be modified in order to render them cleavable by a cleaving agent, for example a protease. In one aspect of the present invention, helper phage may incorporate a coat protein having a cleavable site which, when cleaved, will render the helper phage unable to mediate infection.

Preferred Embodiments

The present invention makes use of cleavable sites in a viral coat protein which is involved in mediating infection and the cleavage of which results in loss of infectivity. The coat protein is selected as the site for cleavage on the grounds that it is available to cleaving agents at the surface of host cells harbouring the virus and at the surface of the virus itself. Thus, virus preparations may be treated with a cleaving agent, in order to render virions having cleavable coat proteins unable to mediate infection of host cells. Alternatively, cells infected with the virus may be treated with a cleaving agent active within the cell, which will prevent packaging of virus encoding a cleavable coat protein.

According to the present invention, reference to selection may be interpreted as a reference to screening, since the same processes may be used to screen phage, as will be apparent to persons skilled in the art.

Cleavable sites may be naturally part of the coat protein, but preferably they are engineered therein. Preferred cleavable sites include protease cleavage sites, which may be found in polypeptides or engineered as an integral part of their sequence. Typically, protease cleavage sites may be defined in terms of amino acid sequences which are susceptible to cleavage by a protease. For example, the invention encompasses the use of protease cleavage sites cleavable by one or more of the proteases trypsin (cleaves at Lys, Arg), chymotrypsin (Phe, Trp, Tyr, Leu), thermolysin (small aliphatic residues), subtilisin (small aliphatic residues), Glu-C (Glu), Factor Xa (Ile/Leu-Glu-Gly-Arg), Arg-C (Arg) and thrombin.

Protease cleavage sites may be incorporated into the coat protein of a virus by constructing a fusion between the coat protein and a further polypeptide, the further polypeptide containing the cleavage site. The further polypeptide should be inserted at a position in the viral coat protein such that it allows the assembly of a functional viral capsid and subsequent infection, but if cleaved will result in the loss of infectivity by the virion.

If the protease cleavage site incorporated in the coat protein remains uncleaved, therefore, the virus is capable of assembly into functional virions and retains the potential to infect host cells. If the protease cleavage site is cleaved, however, the structure of the viral coat protein will be compromised and the viral will lose at least part of its potential to infect host cells.

In a preferred embodiment, the virus for use in the present invention is a bacteriophage, preferably filamentous bacteriophage. Filamentous bacteriophage is widely used in phage display techniques for the selection of polypeptides from phage libraries encoding a large repertoire thereof. Conventionally, the repertoire of polypeptides is inserted in the p3 protein of filamentous bacteriophage, but any other suitable site may be employed within the scope of the present invention.

In the case of the p3 protein of filamentous bacteriophage the protein consists of three domains. The N-terminal D1 is involved in binding to the tolA receptor, D2 in binding to the F-pilus (and mediating infection) and D3 in anchoring the protein to the phage particle (REF). Peptides and proteins can be inserted at the domain boundaries without abolishing infectivity [21, 22], but the presence of all the domains is essential for phage infectivity [23]. The bacteriophage are resistant to proteolysis (allowing their use as "substrate" phage, [19]), but the introduction of polypeptides comprising protease cleavage sites into p3, for example at the junctions between domains leads to loss in infectivity of the phage upon proteolysis.

The protease cleavage sites may be incorporated into heterologous polypeptides, for example those polypeptides encoded in the form of a repertoire in a phage library. As folded polypeptides or proteins are often resistant to proteolysis and unfolded proteins are sensitive, cleavage requires the polypeptide chain to bind and adapt to the specific stereochemistry of the protease active site, and therefore to be flexible, accessible and capable of local unfolding [14, 15]. The cloning of a polypeptide comprising protease cleavage sites at the domain junctions of p3, followed by proteolysis, provides a means of selection for phages bearing proteins that are resistant to proteolysis and are folded.

Furthermore, the use of protease cleavage could be useful for "cleaning up" a repertoire destined for conventional selection according to polypeptide binding characteristics, before selection of the repertoire by binding to solid phase. Thus for a phage antibody library fused at the N-terminus of p3 encoded on a phagemid and rescued with cleavable helper phage, phage antibody fragments which are out of frame with respect to gene 3 could be removed from the libraries by protease cleavage followed by reinfection into bacteria.

This feature may be used, for example, to reduce background in phage display selection experiments. In the case of phage display repertoires (wherein the polypeptide to be selected is cloned at the N-terminus of p3) encoded on phagemid vectors, the use of helper phage comprising a polypeptide comprising protease cleavage sites at the domain

boundaries, followed by proteolysis, provides a means of selection for phages that display the fusion protein by eliminating the helper phage after the "help" has been given.

The use of the protease-cleavable helper followed by protease cleavage selects for phages bearing the fusion protein (and for good expression). As many phages in a repertoire do not display fusion proteins [26] and these contribute to non-specific binding of the phage, this should also improve selection efficiencies. Thus only between 0.1-1% of all phage particles in a phage library carry a gene 3 arising from the phagemid. Therefore the majority (99-99.9 %) of phage particles that have bound non-specifically to the solid support used in selection will only carry p3 from the helper phage (irrespective of the genome carried by the phage particle which most likely will be a phagemid DNA), these particles are rendered non-infective by proteolytic cleavage.

According to a third embodiment, the selection process may be used for identification of interacting protein elements. If two such elements linked by a polypeptide comprising protease cleavage sites are cloned between the D2 and D3 domains for display on phage, the only infectious phages after proteolysis are those in which the D2 and D3 domains are held together by non-covalent interactions between the interacting protein elements. The invention accordingly permits selection of a repertoire of polypeptides for its ability to interact with a selected polypeptide, or a second repertoire of polypeptides. Unlike the two-hybrid system, the invention relies on dissociation of non-interacting elements for the selection step. Moreover, the invention permits the harnessing of the power of phage display to greatly increase the degree of selection.

The invention optionally comprises the use of conditions or agents, during cleavage of the cleavable site, which modulate the lability of the cleavage site in the presence of the cleaving agent. This approach may be used to increase cleavage, for example to select only for polypeptides which fold in such a manner as to closely shield the cleavable site from access by the cleaving agent, or to decrease cleavage, for example to select stable

mutants from a repertoire of polypeptides which is ordinarily relatively labile under cleavage conditions.

For example, modulation of the lability of the cleavable site may be achieved by the use of agents which increase or decrease such lability. Thus, a protein denaturant may be included, at a suitable concentration, to destabilise a polypeptide and render it more labile. Alternatively, a ligand for a polypeptide may be included. The ligand may stabilise the folded structure of the polypeptide, rendering it less sensitive to cleavage. The ligand may, moreover, destabilise the folded structure of the polypeptide, for example by favouring the adoption of an alternative configuration. This may render the polypeptide more accessible to the cleavage agent, and thus more labile.

In a further embodiment, the conditions of the cleavage process may be altered, such as by manipulating the pH or the temperature at which cleavage is conducted, to achieve similar effects. Thus, deviation of the pH from the optimum for the polypeptide comprising the cleavable site may cause the site to become more accessible to the cleaving agent. Similarly, raising (or lowering) the temperature of the conditions under which the polypeptide is cleaved may render the polypeptide more or less susceptible to cleavage.

In some instances, non-covalent interactions may be responsible for peptides retaining their structure and coat proteins remaining viable, even after successful cleavage of the cleavable site. The use of denaturants, temperature variation and other potentially destabilising techniques may also be used to decrease the likelihood of a cleaved polypeptide retaining its structure.

Proteolytic selection for protein folding may be applied in a number of areas, as it allows much larger numbers of proteins to be processed than with conventional screening. For example, it allows the isolation of mutant proteins with improved stability [1], for example from combinatorial libraries of mutants in which residues at several sites are varied simultaneously [39, 40] or from random mutants or by

recombination [3, 4]. It also allows the isolation of novel proteins and architectures from large repertoires of sequences [16-18, 41]; and for improvement in folding stability over several rounds of mutation and increasingly stringent selection, much like the affinity maturation of antibodies.

The invention is further described in the following examples, for the purposes of illustration only.

Materials & Methods

Materials. All restriction enzymes, T4 ligase are obtained from New England Biolabs. Taq DNA polymerase is obtained from HT Biotechnology. Pfu DNA polymerase is obtained from Stratagene. Ultrapure dNTP from Pharmacia. Proteases and the protease inhibitor Pefabloc are obtained from Boehringer Mannheim, except chymotrypsin and trypsin TPCK-treated which are obtained from Sigma. All other chemical are likewise obtained from Sigma.

Phage preparation. *Escherichia coli* TG1 [42] is used for cloning and propagation of phage. TG1 harbouring fd-DOG [43] or derivatives is grown overnight in 2xTY containing 15 μg/ml tetracycline. Phagemids are rescued using KM13 or VCSM13 as described [27]. Phage particles are prepared by two PEG precipitations [44].

Vector construction. The phage vector fd-DOG [43] is used as parent vector for construction of the protease cleavable fd-K108. Unique restriction sites (SfiI, KpnI) are introduced into the glycine rich spacer region between D2 and D3 using the Sculptor *in vitro* mutagenesis system (Amersham) and the oligonucleotide pklinker (Table 4). Further restriction sites (ApaI, SalI) and sequence encoding a protease cleavage site are cloned between the SfiI and KpnI sites using the oligonucleotides polyXafor and polyXaback to create the vector fd-K108.

The protease cleavable helper phage KM13 is prepared from fd-K108 by transplanting into the helper phage VCSM13 a BamH1-ClaI fragment generated by PCR and primers fdPCRBack and LIBSEQfor.

A protease cleavable phagemid vector is derived from fd-K108 much as above except using pCANTAB 3 (Pharmacia). A FLAG-tag is introduced at the N-terminus of D1 by cloning of a NotI-SfiI fragment generated by PCR and primers Flagprimer and LSPAback. To circumvent deletions due to repeated sequence in the D2-D3 linker, the codon usage of the polylinker region is changed in two steps (a) using a Bam-SfiI fragment generated by PCR and primers RECGLYfor and LIBSEQfor, screening recombinants by PCR and the primers LSPAfor and LSPAback, (b) using a KpnI-ClaI fragment generated by PCR and the primers RECGLYback and LIBSEQback, screening recombinants using LSPAfor and LSPAback. The entire p3 gene is sequenced using PCR cycle sequencing with fluorescent dideoxy chain terminators (Applied Biosystems) [45]. The "out of frame" vector pK2 (Fig. 2) is derived from pK1 by site direct mutagenesis using the oligo delCKpn and the Sculptor Amersham kit.

Cloning of Barnase and Villin. The vectors encoding the single barnase mutants, His102->Ala and Leu14->Ala [29, 46] are used as templates for PCR amplification with primers Barnasefor and BarnaseH102Aback and Pfu polymerase. The PCR products (encoding the single mutant His102->Ala, and the double mutant His102->Ala, Leu14->Ala) are digested using the restriction enzymes SfiI and KpnI, and ligated into vector pK2 to give the phagemids pK2BA and pK2BB respectively and the barnase genes sequenced using PCR cycle sequencing.

The 35 amino acid thermostable fragment of the headpiece of the f-actin binding protein villin [33] is amplified from chicken bursa cDNA using PCR primers villinfor and villinback with Pfu polymerase. The PCR products are cloned as above to give the phagemid pK2V.

Resistance of phages to denaturants, pH and proteases. For resistance to denaturants, 10 M urea in PBS (25 mM NaH_2PO_4, 125 mM NaCl pH 7.0) or 8 M GndHCl (Guanidine hydrochloride) and 50 mM Tris-HCl pH 7.4, 1 mM CaCl2 (buffer A) is added to 10 μl phage stocks (10^8-10^{10} TU) to give a volume of 1 ml and the conditions specified in Table 1. The phage are incubated for 1-2 hrs, then 100 μl aliquot added to 1 ml TG1 (OD600 ~ 0.5) and serial dilutions plated on TYE plates with 15 μg/ml tetracycline. For resistance of phage to extremes of pH (2-12), Tris glycine or Tris HCl buffers (0.1 M glycine or 0.1 M Tris respectively) are added to 10 μl phage stocks, and to neutralise each 100 μl aliquot we added 50 μl 1 M Tris-HCl pH 7.4 before infection. For resistance to temperature, buffer A is added to 10 μl phage stocks to give a volume of 1 ml and incubated at a given temperature (20 - 60 C) for 1 hr. 100 μl aliquots are added to TG1 and plated as above. For resistance to proteases, 100 mM NaCl, 50 mM Tris-HCl, 1 mM $CaCl_2$ pH 7.4 (Factor Xa 100 ng/ml or trypsin, chymotrypsin, thrombin, thermolysin and subtilisin all 100 μg/ml) or 50 mM Tris-HCl, 1 mM EDTA pH 7.4 (IgA Protease 10 ng/ml) or 50 mM NH_4CO_3 pH 8.0 (Arg-C 100 μg/ml, Glu-C 100 μg/ml) or 25 mM NaH_2PO_4, 125 mM NaCl pH 7.0 (AspN 40 ng/ml) is added to 10 μl phage stocks (fd-DOG and fd-K108) to give a volume of 100 μl and a final concentration of protease as indicated. Digestions are incubated for 15 min at room temperature, samples (100 μl) are then infected into TG1 as above.

For resistance to proteases in the presence of denaturants samples are prepared as above for urea and temperature denaturation. To 90 μl aliquots 10 μl trypsin (1 mg/ml) is added, after 5 min at room temperature 4 μl Pefabloc (100 mM) is added and the samples are infected into TG1 as above.

Western blot.

Phages (pHEN1 rescued using KM13 and pK1 rescued using VCSM13) are subjected to SDS-PAGE [47] before or after cleavage by trypsin (50 ng/ml). After semidry transfer to PVDF membranes the filter is process essentially as described [27]. The primary

antibody, monoclonal anti-gIII (MoBiTec), is added in a 1:5000 dilution followed by anti-mouse HRP-conjugated antibody (Sigma) in a dilution of 1:50000. Finally the filter is developed using the luminol based Chemiluminescence Western Blotting kit (Boehringer Mannheim).

ELISA

Phage displaying barnase mutants are analysis for binding to the RNase inhibitor barstar as described [44]. 10 pmol biotinylated barstar is mixed with approximately 10^{10} phage displaying barnase mutant A or barnase mutant B or villin or buffer A. Phage binding barstar is captured on Streptavidin coated plates (Boehringer Mannheim) and developed using HRP conjugated anti-M13 antibody (Pharmacia) and 2,2'-Azino-Bis(3-Ethylbenzthiazoline-6-sulfonic acid) (Sigma). Absorbance readings are taken at 405 nm.

Temperature denaturation.

At each temperature approximately 10^{10} phage displaying the barnase mutants or villin (ampicillin resistant) is mixed with a cleavable control fd-K108 (tetracycline resistant), and a non-cleavable control phagemid a chloramphenicol resistant derivative of pHEN1 (P.Wang unpublished), rescued with KM13 in a total volume of 90 μl of buffer A. After equilibration for 20-30 min at the temperature indicated, 10 μl trypsin (5 μg/ml) is added and the incubation continued for 2 min. Trypsin is neutralised by adding 4 μl 100 mM Pefabloc. Infection and serial dilution is performed in TG-1 as above and aliquots are plated on TYE plates containing 100 μg/ml ampicillin + 1% glucose, 30 μg/ml chloramphenicol + 1 % glucose or 15 μg/ml tetracycline.

Selection experiments.

10 μl of serial dilutions of the barnase mutant phage A is mixed with 10 μl of the non-diluted barnase mutant phage B in 70 μl buffer A. After 30 min incubation at 20°C or

37°C 10 μl trypsin (5 μg/ml) is added. Following 2 min. of digestion 4 μl Pefabloc (100 mM) is added. The phage are infected into TG1 as above. A second round of selection are performed by scraping bacteria in 3 ml 2xTY, 50 μl inoculated into 50 ml 2xTY/Amp/Glu and the phagemid rescued and phage prepared as above. Clones are analysed by PCR using the primers LSPAfor and LSPAback followed by restriction digestion using DdeI.

Selections between pK2V and pK1 phage particles are performed as above, except the selection is performed at 10°C. Clones are analysed by PCR using the primers LSPAfor and LSPAback.

Example 1. Resistance of filamentous phage to proteolysis.

Phage is incubated under a range of denaturing conditions *in vitro* and then restored to native conditions immediately before infection of bacteria. The incubation of phage in 10 M urea, or extremes of pH (as low as pH 2, and as high as pH 12) and temperature (as high as 60°C) did not lead to a major loss of infectivity (Table 1). This indicates that the phage is either resistant to denaturing conditions or that if it does unfold it is able to refold rapidly. However with GndHCl a 5 fold loss in phage infectivity is observed above 5 M and a further 5 fold loss at 8 M (Table 1).

Phage is then incubated under native conditions with a range of proteases (trypsin, Factor Xa, IgA protease, Asp-N, chymotrypsin, Arg-C, Glu-C, thrombin, thermolysin, subtilisin) with different specificities. There is no loss in infectivity except for subtilisin which has been reported to cleave the p3 protein [24]. If phage is incubated under denaturing conditions in the presence of proteases such as trypsin in 3.5 M urea (or > 47°C), infectivity is lost. This indicates that under denaturing conditions the unfolding of the phage coat proteins is sufficient to make sites available for proteolysis.

Example 2. Construction of phage with protease cleavage sites.

A sequence (PAGLSEGSTIEGRGAHE) comprising several proteolytic sites is inserted in the flexible glycine-rich region between the D2 and D3 domains of the phage p3. Incubation of the phage (fd-K108) under native conditions with trypsin, thermolysin or subtilisin now resulted in almost complete loss of infectivity (from 10^7 to <10 TU/ml) and incubation with Glu-C and chymotrypsin resulted in a major loss (from 10^7 to 10^4 TU/ml). This indicates that these proteases cleave the new linker. However incubation with Factor Xa, Arg-C or thrombin did not lead to a loss in infectivity, despite the presence of potential cleavage sites for these enzymes. Presumably the presence of the D2 and D3 domains may block access or cleavage for these enzymes in the case of the present polypeptide.

Example 3. Construction of protease cleavable helper phage and phagemid

Fusion of proteins to p3 should lead to multivalent display of the protein on the phage. However if the protein is fused to p3 encoded by a phagemid (such as pHEN1 [25]), and the bacteria harbouring the phagemid is rescued with a helper phage (such as VCSM13), the fusion protein has to compete for incorporation into the phage with the helper p3. This leads to so-called "monomeric" phage, in which usually less than one copy of the fusion protein is attached to each phage particle [26].

The use of "monomeric" phage might be expected to be advantageous for selection of high affinity interactions. Furthermore "monomeric" phage should be more sensitive to proteolysis, as only a single copy of fusion protein need be cleaved for the phage to be rendered non-infectious and as interactions between multimers of fusion protein would be avoided. However a disadvantage is that the majority of infective phages do not display a protein; such phages binding non-specifically to solid phase are amplified during each round of phage growth.

Protease cleavable helper phage are therefore constructed, by introducing the protease cleavage sequence between the D2 and D3 domains to generate the helper phage KM13.

Table 1. Stability of fd-DOG in different denaturing conditions.

Urea (60°C, 90 min)	Control	2 M	4 M	6 M	8 M	10 M	
	0.56	0.64	0.32	0.32	0.80	0.68	
GndHCl (37°C, 90 min)	Control	2 M	4 M	5 M	6 M	7 M	8 M
	0.72	0.60	0.70	0.16	0.13	0.16	0.03
pH (37°C, 30 min)	Control	pH 2.2	pH 4.0	pH 7.4	pH 10	pH 12	
	1.5	0.46	1.3	1.5	1.4	0.40	
Temperature (30 min)	Control	22°C	37°C	60°C			
	9.7	8.3	9.6	12.0			

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Table 1. Stability of wild type fd-DOG under different conditions. The infectivity (TU/ml x 10^{10}) was measured (see Materials and Methods) and has an estimated error of about ± 6%.

KM13 is shown to rescue the phagemid pHEN1. Furthermore trypsin is shown to cleave a major fraction (about 50%) of p3 of the rescued phage as shown by Western blot and detected with an anti-D3 mAb (Fig 1). However phage infectivity is hardly altered by the cleavage; it therefore appears that only a fraction of the p3 need be entire to mediate bacterial infection.

KM13 is also shown to rescue a pHEN1 phagemid encoding a single chain antibody fragment [27]. Here cleavage by trypsin resulted in a 50 fold loss in phage infectivity (data not shown), consistent with indications that only a small fraction of the phage express fusion protein when rescued with helper phage [26, 28].

A protease cleavable phagemid is also constructed. The phagemid can be rescued with KM13 or VCSM13. As expected, infectivity of this phagemid rescued with KM13 (but not VCSM13) is destroyed by trypsin. This phagemid vector is prone to deletions in the D2-D3 linker; by changing the codon usage in the linker regions on either site of the protease cleavable site, and shortening the length of these linker regions, a more stable vector is created(pK1, Fig. 2). In a second vector (pK2), the sequence of the polylinker is arranged so as to place D3 out of frame to render religations within the polylinker non-infectious (Fig. 2).

Example 4. Construction of a phage antibody library using the protease cleavable helper phage.

Bacteria are electroporated with phagemid DNA encoding a repertoire of scFv fragments fused to the N-terminus of p3 (REF) and grown in liquid culture (2xTY containing antibiotic to select for bacteria containing phagemid and glucose to suppress expression of gene 3). In the mid log phase of bacterial growth (OD600 = 0.5) the helper phage KM13 is added to the bacteria to give a ratio of helper phage to bacteria of 20:1. The bacteria is incubated at 37°C without shaking for 45 min then with shaking for 45 min. The bacteria is harvested by centrifugation and resuspended in fresh medium containing 50 μg/ml kanamycin and antibiotic to select for presence of

phagemid DNA, glucose is NOT added to allow the promoter in front of fusion gene3 to be functional (leaky). The culture is grown overnight at 30°C with shaking.

Bacteria is removed from the phage containing supernatant by centrifugation. Phage is precipitated from the supernatant by adding 1/5 the volume of 20%PEG/ 2.5 M NaCl. After 1-2 hours at 4°C the precipitated phage is collected by centrifugation. The phage is resuspended in PBS (a second PEG precipitation is optional) and can be used in selection.

The library of phages is allowed to bind to antigen (immobilised on solid support such as an immunotube or in solution to tagged (i.e. biotinylated) antigen which can be immobilised after affinity binding of phage antibodies). Unbound phage is removed by extensive washing (the stringency of washing can be varied with respect to time and detergents added).

Phage libraries in which a cleavable tag, such as with the c-myc tag inserted between the antibody and gene 3 can be eluted by addition of trypsin in solution at a concentration of 0.1 to 1 mg/ml. (Phage libraries without a cleavable sequence between the antibody and gene 3 can be eluted by adding i.e.. 100 mM Triethylamine. In this case the solution is neutralised by adding 1 M Tris-HCl pH 7.4. and after 10 min, trypsin added to a final concentration of 0.1 to 1 mg/ml.) Trypsin also cleaves the copies of gene 3 from the helper phage, while leaving gene 3 from the phagemid intact, thus phage carrying an antibody fusion will be infective.

Phage is used to infect bacteria in mid log phase of growth (OD600 = 0.5), and the bacteria is plated on agar plates containing antibiotic selecting for phagemid DNA. Individual clones were picked and phage prepared as above. The resulting phage is used in ELISA to identify phage antibodies binding specifically to the antigen of interest.

Example 5. Selection for folding using barnase as a model.

Barnase is a small RNAse of 110 amino acid residues whose folding has been extensively studied (for review [2]). Barnase contains multiple sites for trypsin cleavage, although the folded protein is resistant to cleavage (data not shown). Phage with barnase cloned between D2 and D3 should therefore be resistant to protease cleavage and capable of selection.

As barnase is toxic to *Escherichia coli*, a mutant A (His102->Ala) is cloned which is catalytically inactive but stable [29, 30] into the phagemid pK2. A mutant B (His102->Ala,Leu14->Ala) is also cloned, with lower stability; Leu14 is buried in the hydrophobic core and its mutation creates a large cavity in the core affecting the packing of different structural elements [31]. The phages (rescued with KM13) are shown to bind to the inhibitor barstar by ELISA, and therefore display the mutant barnases in a folded form (Fig. 3).

The phages are then incubated with trypsin at a range of temperatures (Fig. 4). After incubation at 10°C, there is a decrease in phage infectivity of 5 to 10 fold for both mutants, suggesting that (as above with the display of scFv fragment), that only a small fraction of the phages display the fusion protein. There is no further loss in infectivity on cleavage until 30°C (for mutant B) or 37°C (for mutant A). In both cases the major transition is at least 10°C below that expected for the reversible thermal unfolding of the mutants.

Phages A and B are mixed in different ratios, and incubated the mixture at 20°C with trypsin where both mutants are stable to cleavage, or at 37°C where only A is stable. After "proteolytic selection" the phages are plated and analysed by PCR followed by restriction digest which distinguishes the mutants. As shown in the Table 2, after a single round of selection at 37°C, mutant A is enriched by a factor of 1.6×10^4, and after two rounds by 1.3×10^6. No enrichment can be detected at 20°C.

Example 6. Selection for folding using villin as a model.

The 35 amino acid subdomain of the headpiece domain of the f-actin-bundling protein villin [32] us much smaller than barnase, but is stable to temperature and to proteolysis; furthermore its stability does not rely on disulphide bonds or binding ligands [33]. The villin subdomain (which contains several potential trypsin cleavage sites) is cloned between the D2 and D3 domains of the phage, and incubated with trypsin at different temperatures (Fig. 4). The profile for loss of infectivity is not as sharp as with barnase, with the major transition below 35°C, considerably below the thermal unfolding of villin (70°C) [32, 33]. The phage displaying villin are mixed with pK1 and incubated with trypsin. After a single round of proteolytic selection, the fusion phage are enriched 8.7×10^3 fold (Table 3).

In summary, the results from Examples 1 and 2 show that the infectivity of the phage is relatively resistant to temperature, pII, urea and GndHCl, and to several proteases, but if a flexible linker comprising a protease cleavage site is inserted between domains D2 and D3 of the phage coat protein p3, the phage becomes sensitive to cleavage. By contrast, as shown in Examples 5 and 6, if the protease cleavage sites comprise a folded protein domain such as barnase or villin, the phage is resistant to cleavage. This allows proteolytic selection for protein folding, with enrichment factors of greater than 10^4 fold for a single round of selection. Selection is evident for both for barnase, an average sized [34] domain of 110 amino acids and for villin, a small domain of 35 amino acids.

Discrimination between structures of different stabilities can be accomplished by increasing the stringency of proteolytic selection. Thus with increase in temperature, both barnase and villin became susceptible to cleavage, reflecting protein unfolding. However the main impact of protease cleavage is at a temperature lower than the unfolding transition as measured by circular dichroism [38]. This may reflect the fact that the unfolding transition is a fully reversible process, whereas the cleavage by proteases (of unfolded structure) is a kinetic and irreversible process, pulling over the

equilibrium from folded to unfolded (and cleaved) structure. This is consistent with the CD unfolding transition seen with villin [33], where at temperatures as low as 35°C there is evidence of unfolding, the same point at which villin starts to become susceptible to protease attack.

Table 2 Selection of Barnase mutants

		Phage A: Phage B				
		1:1[a]	$1:10^{-2}$	$1:10^{-4}$	$1:10^{-6}$	$1:10^{-8}$
Round 1	Phage A	16 (14[b])	24	20	0	nd
	Phage B	8 (10[b])	0	4	24	nd
	Enrichment	-	-	$1.6x10^4$	-	nd
Round 2	Phage A	nd	nd	nd	24	0
	Phage B	nd	nd	nd	12	36
	Enrichment	nd	nd	nd	$1.3x10^6$	-

Mixtures of barnase mutants (A+B) in ratios from 1:1 to $1:10^{-8}$ were selected by proteolysis at 37 °C, 24 (or 36 in round 2) phage clones analysed and numbers of each mutant noted above. [a] Selection at 20 °C where both mutants are expected to be stable, [b] before selection.

Table 3 Selection of villin

	pK1 : villin-phage			
	1:1	$1:10^{-2}$	$1:10^{-4}$	$1:10^{-6}$
pK1	0 (16[a])	0	7	24
Villin	24 (8[a])	24	17	0
Enrichment	-	-	$8.7x10^3$	-

Mixtures of villin-phage and pK1 rescued with KM13 in ratios from 1:1 to $1:10^{-6}$ were selected by proteolysis at 10°C, 24 phage clones analysed and number of each noted above. [a] before selection.

Table 4. Primer sequences

pklinker	5'GGCACCCTCAGAACGGTACCCCACCCTCAGAGGCCGGCTGGGCCGCCACCCTCAG AG 3'
polyXafor	5'GGTGGCGGCCCAGCCGGCCTTTCTGAGGGGTCGACTATAGAAGGACGAGGGCCCA GCGAAGGAGGTGGGGTACCCCCTTCTGAGGGTGG 3'
polyXaback	5'CCACCCTCAGAAGGGGGTACCCCACCTCCTTCGCTGGGCCCTCGTCCTTCTATAG TCGACCCCTCAGAAAGGCCGGCTGGGCCGCCACC 3'
fdPCRBack	5'GCGATGGTTGTTGTCATTGTCGGC 3'
LIBSEQfor	5'AAAAGAAACGCAAAGACACCACGG 3'
LIBSEQback	5'CCTCCTGAGTACGGTGATACACC 3'
LSPAfor	5'GTAAATTCAGAGACTGCGCTTTCC 3'
LSPAback	5'ATTTTCGGTCATAGCCCCCTTATTAG 3'
Flagprimer	5'CAAACGGGCGGCCGCAGACTACAAGGATGACGACGACAAGGAAACTGTTGAAAGT TGTTTAGCAA 3'
RECGLYfor	5'CCCCTCAGAAAGGCCGGCTGGGCCGCCGCCAGCATTGACAGGAGGTTCAGG 3'
RECGLYback	5'GAAGGAGGTGGGGTACCCGGTTCCGAGGGTGGTTCCGGTTCCGGTGAT TTTG 3'
delCKpn	5'CCCTCGGAACCGGTACCCCAGCTGCTTCGTGGGCCC 3'
Barnasefor	5'CTGGCGGCGGCCCAGCCGGCCCTGCACAGGTTATCAACACGTTTGAC 3'
BarnaseH102Aback	5'CTCGGAACCGGTACCTCTGATTTTTGTAAAGGTCTGATAAGCG 3'
villinfor	5'GGCGGCCCAGCCGGCCTTTCTCTCTCTGACGAGGACTTCAAGGC 3'
villinback	5'CCTCGGAACCGGTACCGAAGAGTCCTTTCTCCTTCTTGAGG 3'

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Claims:

1. A method for the selection of a virus comprising the steps of:

(a) introducing a polypeptide comprising a cleavable site into the sequence of a viral coat protein; and

(b) exposing the virus to a cleaving agent such that the cleavable site is cleaved and the viral coat protein is unable to mediate infection of the virus.

2. A method according to claim 1, further comprising the step of :

(c) selecting those viruses which remain infective.

3. A method according to claim 1 or claim 2 in which the virus encodes a repertoire of sequences.

4. A method according to any preceding claim in which the virus displays a heterologous peptide or protein on its surface.

5. A method according to claim 4 in which the repertoire of sequences encodes the displayed heterologous peptide or protein.

6. A method according to any one of claims 3 to 5 in which the cleavable site is comprised within the repertoire of sequences.

7. A method according to claim 4 in which the displayed peptide or protein is introduced within the sequence of a coat protein of the virus.

8. A method according to any one of claims 3 to 7 in which the nucleic acid encapsidated by the virus comprises a repertoire of sequences.

9. A method according to any one of claims 3 to 8 in which the cleavable site is comprised within the repertoire of sequences.

10. A method according to any one of claims 3 to 9 in which members of the repertoire that are resistant to cleavage are propagated by infection.

11. A method according to claim 10 in which those virions resistant to cleavage display folded proteins or polypeptides.

12. A method according to claim 11 in which the cleavage is undertaken under conditions at which some members of the repertoire are at least partially unfolded.

13. A method of claim 10 in which the proteolysis is undertaken in the presence of molecules which stabilise or destabilise the displayed polypeptide.

14. A method of claim 13 in which the proteolysis is undertaken in the presence of protein denaturants.

15. A method of claim 13 in which the proteolysis is undertaken in the presence of a ligand for the folded protein.

16. A method according to any preceding claim for the isolation of a protein or polypeptide with improved stability.

17. A method according to any one of claims 3 to 16 in which the repertoire of displayed proteins are selected by binding to a ligand.

18. A method according to any preceding claim in which the virus is a bacteriophage.

19. A method according to claim 18 in which the coat protein is that protein encoded by gene 3 of a filamentous bacteriophage.

20. A method according to claim 19 in which the cleavage sites are introduced between the second and third domain of the gene 3 protein.

21. A method according to claim 20 wherein the bacteriophage are helper bacteriophage used in conjunction with phagemids.

22. A method according to claim 18 in which the encapsidated nucleic acid of the bacteriophage is a phagemid and requires the use of a helper bacteriophage.

23. A method according to any preceding claim, wherein the cleavable site is a protease cleavable site, and the cleaving agent is a protease.

24. A method comprising the steps of:

(a) creating a library of phagemids encoding a repertoire of heterologous polypeptides;

(b) expressing the phagemids in the presence of helper phage;

(c) subjecting the phage to protease cleavage;

(d) using the phage to infect bacteria; and

(e) isolating the phagemids.

Abstract

The present invention concerns a method for the selection of a virus comprising the steps of:

(a) introducing a polypeptide comprising a cleavable site into the sequence of a viral coat protein; and

(b) exposing the virus to a cleaving agent such that the cleavable site is cleaved and the viral coat protein is unable to mediate infection of the virus.

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